In the Application of: John E. Sims and Blair R. Renshaw | Docket No.: 2932-B

Serial No.: 09/976,472 | Group Art Unit: 1646

Filing Date: October 11, 2001 | Examiner: J. Andres

For: IL-1 eta DNA and Polypeptides

DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop AF
Commissioner for Patents
P. O. Box 1540
Alexandria, VA 22313-1450

Sir:

I, John E. Sims, do hereby declare as follows:

1. I am currently employed as a Distinguished Fellow in the Department of Molecular Immunology of Immunex Corporation, a wholly-owned subsidiary of Amgen Inc. I received a Ph.D. degree from Harvard University in Biochemistry; a copy of my curriculum vitae is attached. I am a co-inventor of the subject matter disclosed and claimed in the above-identified patent application.

2. As described in the attached Sims et al. article (*TRENDS in Immunology*, 22(10):536; 2001), since the time the original application was filed disclosing and claiming IL-1 eta polynucleotides and polypeptides, a new nomenclature has been implemented for the IL-1 family. Under that new nomenclature system, IL-1 eta is referred to as IL-1F8 (i.e., the eighth member of the IL-1 family). I use that nomenclature throughout this Declaration, but I affirm that IL-1 eta and IL-1F8 are the same protein.

3. I prepared or directed the preparation of an expression vector used to express IL-1 F8 as a glutathione S-transferase (GST) fusion protein in *E. coli*. The fusion protein was purified and used in several experiments to analyze its activity.

4. In one such experiment, NCI/ADR-RES cells were plated into 24-well dishes, and stimulated on the following day with IL-1F8. Supernatants were collected 48hrs after addition of the IL-1F8 and analyzed for the presence of various cytokines (IL-1beta, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p70), TNF-alpha, IFN-gamma and GM-CSF) using a commercially available cytokine detection system (the Beadlyte™ human multi-cytokine detection system 3 from Upstate Cell Signaling Solutions, Lake Placid, NY) as per manufacturer's instructions. Plates were read on a Luminex^{100} LabMAP ™ System (Luminex Corporation, Austin, TX) and results analyzed using Masterplex QT software (MiraBio Inc., Alameda, CA).

5. The results indicated that IL-1F8 induced the production of IL-6, IL-8 and some GM-CSF at 48 hours of treatment, confirming its activity as a proinflammatory cytokine.

6. In other experiments, IL-1F8 was shown to activate NFkappaB (nuclear factor kappaB, a transcription factor that is a hallmark of an immune and/or inflammatory response), as well as the MAPKs (mitogen-activated protein kinases) JNK (c-*jun* N-terminal kinase) and ERK1/2 (extracellular signal-regulated kinase 1/2) in NCI/ADR-RES cells.

7. Induction of proinflammatory cytokines and activation of NFkappaB and MAPKs are activities that IL-1F8 shares with other proinflammatory members of the IL-1 family, such as IL-1alpha and IL-1beta. I concluded from these experiments that IL-1F8 is capable of activating responses that enhance immune responses and promote inflammation, and thus plays a role in the immune system and in the pathogenesis of inflammatory diseases.

8. IL-1alpha, IL-1beta and other proinflammatory members of the IL-1 family have served as targets in testing and identifying antagonists thereof for use in treating inflammatory conditions, as shown in the attached articles by Dayer et al. and Debets et al. (Dayer et al., *Curr. Opin. Rheumatol.* 13:170, 2001; Debets et al., *J. Immunol.* 165:4950, 2000). Thus, the fact that IL-1F8 shares the ability to induce production of proinflammatory cytokines and activate NFkappaB and MAPKs with other proinflammatory members of the IL-1 family confirms that IL-1F8 polypeptides will be useful in a screening assay for compounds and small molecules which inhibit activation by (antagonize) IL-1F8 polypeptide, as disclosed in the above referenced patent application (see, for example, the disclosure at page 20, line 36 through page 21, line 4 of the present application).

9. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: _____
John E. Sims, Ph.D.

Dated: 12/8/03

Attachments: 4

el042601 12/4/03

Curriculum Vitae

John Ernest Sims

Address: Immunex Corporation
51 University Street
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Date of Birth: March 11, 1947

Citizenship: USA

Education

1965-1969 Harvard College, Cambridge, Massachusetts
Degree: B.A. cum laude, June 1969

1969-1970 University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania

1970-1979 Harvard University, Department of Biochemistry
and Molecular Biology, Cambridge, Massachusetts
Degree: Ph.D., June 1979

1979-1983 Post-doctoral fellow, laboratories of T. Rabbitts
and C. Milstein, MRC Laboratory of Molecular Biology, Cambridge, England

Employment

1983-1984 Staff, Medical Research Council Laboratory of Molecular Biology, Cambridge, England

1984-1988 Staff Scientist, Molecular Biology Department,
Immunex Corporation, Seattle, Washington

1989-1990 Senior Staff Scientist,
Immunex Corporation, Seattle, Washington

1990-1992 Head, Division of Gene Discovery
Department of Molecular Biology
Immunex Corporation, Seattle, Washington

1993-2000 Director
Department of Molecular Genetics
Immunex Corporation, Seattle, Washington

2000-2002 Senior Director
Department of Molecular Genetics
Immunex Corporation, Seattle, Washington

2002	Vice President Department of Molecular Biology Immunex Corporation, Seattle, Washington
2002- Present	Distinguished Fellow Molecular Immunology Amgen, Seattle, Washington

Activities

Editorial Board, Journal of Interferon & Cytokine Research, 1991-Present

Ad hoc reviewer for journals:

Analytical Biochemistry, Brain Research, Circulation, Clinical & Experimental Metastasis, Cytokine, Endocrinology, European Cytokine Network, European Journal of Cell Biology, European Journal of Immunity, Human Molecular Genetics, Immunity, International Journal of Immunopharmacology, International Journal on Genes & Genomes, Journal of Clinical Investigation, Journal of Immunology, Journal of Molecular Biology, Laboratory Investigation, Life Sciences, Molecular Immunology, PNAS, Virology

Ad hoc reviewer for grants:
Welcome Trust, Canadian MRC, NIH (Allergy, Immunological Sciences Study Sections)
Arthritis Research Council, United Kingdom

Patents

U.S. Patent 5,296,592 (2001-F)
Process for Purifying Interleukin-1 Receptors

U.S. Patent 5,319,071 (2001-E)
Soluble Interleukin-1 Receptors

U.S. Patent 5,350,683 (2003-D)
DNA Encoding Type II Interleukin-1 Receptors

U.S. Patent 4,968,607 (2001-A)
Interleukin-1 Receptors

U.S. Patent 5,081,228 (2001-B)
Interleukin-1 Receptors

U.S. Patent 5,180,812 (2002)
Soluble Human Interleukin-1 Receptors, Compositions and Method of Use

Patents (Continued)

U.S. Patent 5,422,248
DNA Sequences Encoding Granulocyte-Colony Stimulating Factor Receptors

U.S. Patent 5,464,937
Type II Interleukin-1 Receptors

U.S. Patent 5,488,032
Method of Using Soluble Human Interleukin-1 Receptors to Suppress Inflammation

U.S. Patent Re 3540
Soluble Human Interleukin-1 Receptors, Compositions and Method of Use

U.S. Patent 5,492,888
Method of Using Soluble Human Interleukin-1 Receptors to Suppress Immune Responses

U.S. Patent 5,576,191
Cytokine that Binds ST2

U.S. Patent 5,589,456
Granulocyte-Colony Stimulating Factor Receptors

U.S. Patent 5,767,064
Soluble Type II Interleukin-1 Receptors and Methods

U.S. Patent 5,776,731
DNA Encoding Type-I Interleukin-1 Receptor-Like Protein Designated 2F1

U.S. Patent 6,080,557
IL-1/TNF-α-Activated Kinase (ITAK, and Methods of Making and Using the Same)

U.S. Patent 6,090,918
Receptor Protein Designated 2F1

U.S. Patent 6,451,760 B1
Treatment of Inflammation Using A 2F1 Polypeptide Or An Antibody Thereto

U.S. Patent 6,541,232 B1
Polypeptides Having Kinase Activity

U.S. Patent 6,511,665 B1
Antibodies to Interleukin-1 Receptors

U.S. Patent 6,555,520 B2
Human TSLP DNA and Polypeptides

U.S. Patent 6,521,740 B1
Type II Interleukin-1 Receptors

U.S. Patent 6,589,764 B1
IL-18 Receptor Fusion Proteins

Published Patent Cooperation Treaty (PCT) Applications

PCT Serial No. PCT/US88/03926
Publication No. WO 89/04838
Interleukin-1 Receptors

PCT Serial No. PCT/US90/05434
Publication No. WO 91/05046
Granulocyte-Colony Stimulating Factor Receptors

PCT Serial No. PCT/US91/03498
Publication No. WO 91/18982
Type II Interleukin-1 Receptors

PCT Serial No. PCT/US97/00690
Publication No. WO 97/25347
IL-1 Receptor Interacting Protein

PCT Serial No. PCT/US97/01697
Publication No. WO 97/31010
Receptor Protein Designated 2F1

PCT Serial No. PCT/US97/08516
Publication No. WO 97/47750
IL-1/TNF-alpha-Activated Kinase (ITAK, and Methods of Making and Using the Same)

PCT Serial No. PCT/US98/27368
Publication No. WO 99/32626
SIGIRR DNA and Polypeptides

PCT Serial No. PCT/US98/27625
Publication No. WO 99/32629
TIGIRR DNA and Polypeptides

PCT Serial No. PCT/US99/00514
Publication No. WO 99/35268
IL-1 Delta DNA and Polypeptides

PCT Serial No. PCT/US99/01420
Publication No. WO 99/37773
ACPL DNA and Polypeptides

PCT Serial No. PCT/US99/01419
Publication No. WO 99/37772
IL-18 Receptors

PCT Serial No. PCT/US99/23533
Publication No. WO 00/20595
Interleukin-1 Homolog

Research Publications

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2. Sims, J., Koths, K. and Dressler, D. 1978. Single-stranded phage replication: Positive- and negative-strand DNA synthesis. Cold Spring Harbor Symposium 43, 349-365.

3. Sims, J., Capon, D. and Dressler, D. 1979. dnaG (Primase)-dependent origins of DNA replication: Nucleotide sequences of the negative strand initiation sites of bacteriophages St-1, ϕK and α3. J. Biol. Chem. 254, 12615-12628.

4. Sims, J. and Benz, E. 1980. Initiation of DNA replication by the Escherichia coli dnaG protein: Evidence that tertiary structure is involved. Proc. Natl. Acad. Sci. USA 77, 900-904.

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7. Sims, J., Tunnacliffe, A., Smith, W.J. and Rabbitts, T.H. 1984. Complexity of human T-cell antigen receptor β-chain constant- and variable-region genes. Nature 312, 541-545.

8. Rabbitts, T.H., Lefranc, M.P., Stinson, M.A., Sims, J.E., Schroder, J., Steinmetz, M., Spurr, N.L., Solomon, E. and Goodfellow, P.N. 1985. The chromosomal location of T-cell receptor genes and a T cell rearranging gene: Possible correlation with specific translocations in human T cell leukemia. EMBO J. 4, 1461-1465.

9. Tunnacliffe, A., Sims, J.E. and Rabbitts, T.H. 1986. T3δ pre-mRNA is transcribed from a non-TATA promoter and is alternatively spliced in human T cells. EMBO J. 5, 1245-1252.

10. Sims, J.E., March, C.J., Cosman, D., Widmer, M.B., MacDonald, H.R., McMahan, C.J., Grubin, C.E., Wignall, J., Jackson, J.L., Call, S.M., Friend, D., Alpert, A., Gillis, S., Urdal, D.L. and Dower, S.K. 1988. cDNA expression cloning of the receptor for interleukin-1, a member of the immunoglobulin superfamily. Science 241, 585-589.

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12. Curtis, B.M., Gallis, B., Overell, R., McMahan, C.J., de Roos, P., Eisenman, J., Dower, S.K. and Sims, J.E. 1989. The T cell IL-1 receptor cDNA expressed in CHO cells regulates functional responses to IL-1. Proc. Natl. Acad. Sci. USA 86, 3045-3049.

Research Publications (Continued)

13. Dower, S.K., Wignall, J., Schooley, K., McMahan, C.J., Jackson, J., Prickett, K.S., Lupton, S., Cosman, D. and Sims, J.E. 1989. Retention of ligand binding activity by the extracellular domain of the IL-1 receptor. J. Immunol. 142, 4314-4320.

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15. Bomsztyk, K., Sims, J.E., Stanton, T.H., Slack, J., McMahan, C.J., Valentine, M.A. and Dower, S.K. 1989. Evidence for different interleukin 1 receptors in murine B- and T-cell lines. Proc. Natl. Acad. Sci. USA 86, 8034-8038.

16. Gallis, B., Prickett, K.S., Jackson, J., Slack, J., Schooley, K., Sims, J.E. and Dower, S.K. 1989. Interleukin-1 induces rapid phosphorylation of the IL-1 receptor. J. Immunol. 143, 3235-3240.

17. Sims, J.E., Acres, R.B., Grubin, C.E., McMahan, C.J., Wignall, J.M., March, C.J. and Dower, S.K. 1989. Cloning of the interleukin-1 receptor from human T cells. Proc. Natl. Acad. Sci. USA 86, 8946-8950.

18. Slack, J., Sims, J.E., Pitt, A.M. and Dower, S.K. 1989. Application of the multiscreen system to cytokine radioreceptor assays. BioTechniques 7, 1132-1138.

19. Fanslow, W.C., Sims, J.E., Sassenfeld, H., Morrissey, P.J., Gillis, S., Dower, S.K. and Widmer, M.B. 1990. Regulation of alloreactivity in vivo by a soluble form of the interleukin-1 receptor. Science 248, 739-742.

20. Copeland, N.G., Silan, C.M., Kingsley, D.M., Jenkins, N.A., Cannizzaro, L.A., Croce, C.M., Huebner, K. and Sims, J.E. 1990. Chromosomal location of murine and human IL-1 receptor genes. Genomics 9, 44-50.

21. Munoz, E., Zubiaga, A.M., Sims, J.E. and Huber, B.T. 1990. IL-1 signal transduction pathways. I. Two functional IL-1 receptors are expressed in T cells. J. Immunol. 146, 136-143.

22. Spriggs, M., Lioubin, P., Slack, J. Cosman, D., Sims, J. and Bauer, J. 1990. Induction of an interleukin-1 receptor (IL-1R) on monocytic cells. Evidence that the receptor is not encoded by a T cell-type IL-1R mRNA. J. Biol. Chem. 265, 22499-22505.

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Research Publications (Continued)

24. Ostrowski, J., Sims, J., Sibley, C., Valentine, M., Dower, S., Meier, K., and Bomsztyk, K. 1991. A serine/threonine kinase activity is closely associated with a 65-kDa phosphoprotein specifically recognized by the κB enhancer. J. Biol. Chem. 266, 12722-12733.

25. Bird, T.A., Woodward, A., Jackson, J.L., Dower, S.K. and Sims, J.E. 1991. Phorbol ester induces phosphorylation of the 80 kilodalton murine interleukin 1 receptor at a single threonine residue. Biochem. Biophys. Res. Comm. 177, 61-67.

26. McMahan, C.J., Slack, J.L., Mosley, B., Cosman, D., Lupton, S.D., Brunton, L.L., Grubin, C.E., Wignall, J.M., Jenkins, N.A., Brannan, C.I., Copeland, N.G., Huebner, K., Croce, C.M., Cannizzarro, L.A., Benjamin, D., Dower, S.K., Spriggs,M.K. and Sims, J.E. 1991. A novel IL-1 receptor, cloned from B cells by mammalian expression is expressed in many cell types. EMBO J. 10, 2821-2832

27. Spriggs, M.K., Nevens, P.J., Grabstein, K., Dower, S.K., Cosman, D., Armitage, R.J., McMahan, C.J. and Sims, J.E. 1992. Molecular characterization of the interleukin-1 receptor (IL-1R) on monocytes and polymorphonuclear cells. Cytokine 4, 90-95.

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30. Bird, T.A., Schule, H.D., Delaney, P.B., Sims, J.E., Thoma, B., and Dower, S.K. 1993. Evidence that MAP (Mitogen-Activated Protein) kinase activation may be a necessary but not sufficient signal for a subset of responses in IL-1-treated epidermoid cells. Cytokine 4, 429-440.

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32. Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., Giri, J.G., Dower, S.K., Sims, J.E. and Mantovani, A. 1993. The type II receptor as a decoy target for IL-1 regulated by interleukin-4. Science 261, 472-475.

33. McKean, D.J., Podzorski, R.P., Bell, M.P., Nilson, A.E., Huntoon, C.J., Slack, J., Dower, S.K. and Sims, J. 1993. Murine T helper cell-2 lymphocytes express type I and type II IL-1 receptors, but only the type I receptor mediates costimulatory activity. J. Immunol. 151:3500.

Research Publications (Continued)

34. Sims, J.E., Gayle, M.A., Slack, J.L., Alderson, M.R., Bird, T.A., Giri, J.G., Colotta, F., Re, F., Mantovani, A., Shanebeck, K., Grabstein, K.H. and Dower, S.K. 1993. Interleukin-1 signalling occurs exclusively via the type I receptor. Proc. Natl. Acad. Sci. USA 90, 6155-6159.

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36. Gayle, M.A., Sims, J.E., Dower, S.K. and Slack, J.L. 1994. Monoclonal antibody 1994:01 (also known As Alva 42) reported to recognize Type II IL-1 receptor is specific for HLA-DR alpha and beta chains. Cytokine. 4:1, 83-86.

37. Arend, W. P., Malyak, M., Smith, M. F. Jr., Whisenand, T. D., Slack, J. L., Sims, J. E., Giri, J. G., Dower, S. K. 1994. Binding of IL-1α, IL-1β, and IL-1 receptor antagonist by soluble IL-1 receptors and levels of soluble IL-1 receptors in synovial fluids. J. Immunol. 153:10, 4766-4774.

38. Giri, J.G., Wells, J., Dower, S.K., McCall, C.E., Guzman, R.N., Slack, J., Bird, T.A., Shanebeck, K., Grabstein, K.H., Sims, J.E., and Alderson, M.R. 1994. Elevated levels of shed type II IL-1 receptor in sepsis: potential role for Type II receptor in regulation of IL-1 responses. J. Immunol. 153:12, 5802-5809.

39. Groves, R.W., Giri, J., Sims, J., Dower, S.K., and Kupper, T.S. 1995. Inducible expression of type 2 IL-1 receptors by cultured human keratinocytes. J. Immunol. 154:8, 4065-4072.

40. Sims, J., Painter, S., Gow, I. 1995. Genomic organization of the Type I and Type II IL-2 receptors. Cytokine. 7:6, 483-490.

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43. Gayle, M.A., Slack, J.L., Bonnert, T.P., Renshaw, B.R., Sonoda, G., Taguchi, T., Testa, J.R., Dower, S.K., and Sims, J.E. 1996. Cloning of a putative ligand for the T1/ST2 receptor. J. Biol. Chem. 271, 5784-5789.

44. Taguchi, T., Mitcham, J.L., Dower, S.K., Sims, J.E., and Testa, J.R. 1996. Chromosomal localization of TIL, a gene encoding a protein related to the drosophila transmembrane receptor toll, to human chromosome 4p14. Genomics 32, 486-488.

Research Publications (Continued)

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46. Colotta, F., Saccani, S., Giri, J.G., Sims, J.E., Introna, M., and Mantovani, A. 1996. Regulated expression and release of the Interleukin 1 (IL-1) decoy receptor in human mononuclear phagocytes. J. Immunol. 156, 2534-2541.

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Research Publications (Continued)

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A new nomenclature for IL-1-family genes

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The past two years have seen the discovery of six novel members of the interleukin-1 (IL-1) gene family[1–6] (see E. Dunn *et al.*, pp. 533–536). The designation of these novel genes as relatives of the previously known family members encoding IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra) and IL-18 is based on their conservation of amino acid sequence, identity of gene structure, and (probable) adoption of the same three-dimensional fold. Unfortunately, there are as many different systems of nomenclature for the novel genes as there are groups that have cloned them – a situation that has led to much

Table 1. Proposed nomenclature for the IL-1 family[a]

Protein name	GenBank® accession number	Refs	Previous names	Splice variant	GenBank® accession number	Refs	Human gene symbol	Mouse gene symbol
(IL-1F1)	NM_000575	–	IL-1α	–	–	–	*IL1A*	*Il1a*
(IL-1F2)	NM_000576	–	IL-1β	–	–	–	*IL1B*	*Il1b*
(IL-1F3)	NM_000577	–	IL-1ra	IL-1F3b	M55646	–	*IL1RN*	*Il1rn*
				IL-1F3c	X84348	–	–	–
(IL-1F4)	NM_001562	–	IL-18, IGIF, IL-1γ	–	–	–	*IL18*	*Il18*
IL-1F5	AF186094	1	IL-1Hy1, FIL1δ, IL-1H3, IL-1RP3, IL-1L1 and IL-1δ	IL-1F5b	AJ242738	4	*IL1F5*	*Il1f5*
IL-1F6	AF201831	2	FIL1ε	–	–	–	*IL1F6*	*Il1f6*
IL-1F7	AF201832	2	FIL1ζ, IL-1H4, IL-1RP1 and IL-1H	IL-1F7b	AF200496	3	*IL1F7*	*Il1f7*
				IL-1F7c	AF251120	5	–	–
IL-1F8	AF201833	2	FIL1η and IL-1H2	IL-1F8b	AF200494	3	*IL1F8*	*Il1f8*
IL-1F9	AF200492	3	IL-1H1, IL-1RP2 and IL-1ε[b]	–	–	–	*IL1F9*	*Il1f9*
IL-1F10	AF334755	6	IL-1Hy2 and FKSG75	IL-1F10b	AY026753	–	*IL1F10*	*Il1f10*

[a]Abbreviations: FIL1, family of IL-1; IGIF, interferon γ inducing factor; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IL1-RP3, IL-1 related protein 3; IL-1L1, IL-1 like 1; IL-1Hy1, IL-1HySeq1; IL-1H3, IL-1 homolog 3.
[b]Although GenBank® AF206696 (termed IL-1ε) is identical to IL-1F9, the corresponding mouse entry (AF206697) might be more closely related to IL-1F6 than to IL-1F9.

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confusion and that we hope now to rectify. We propose a unified system of nomenclature for the novel genes, as set out in Table 1. This system acknowledges their relationship to the previously known members, although it is not our intention to change common usage of the names IL-1α, IL-1β, IL-1ra and IL-18.

We suggest that the novel genes of the IL-1 family be given the designation *IL1F5–IL1F10*, in order of their date of publication. The '*IL1*' portion of the name is maintained to indicate the evolutionary relationship to the traditional types of IL-1, and the letter '*F*' (for family) is interpolated to make it clear that the new products are not simply functional duplicates of IL-1α and IL-1β. Protein products are named in the style of 'IL-1F5', following the style used generally for the classical types of IL-1. Many of these genes yield multiple, alternatively spliced mRNAs; the different splice forms and their protein products are indicated by appending a lower case letter (e.g. IL-1F7a), again in order of date of publication.

We expect that the adoption of this new nomenclature will aid the clarity of exposition and understanding as functions for these new gene products are elucidated.

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Meeting Report

Designing novel therapeutic strategies for rheumatic diseases

Florence Apparailly, Steffen Gay and Christian Jorgensen

The Second International Meeting of Innovative Rheumatology: Gene and Cell Therapies of Arthritis and Related Disorders was held in Montpellier, France, from 17–18 May 2001.

Exciting new data have emerged that provide new concepts to explain the puzzling role of cytokines, transcription factors, adhesion molecules and apoptosis-regulating molecules in the pathogenesis of rheumatoid arthritis (RA). Based on the fact that synovial fibroblasts appear to be activated in RA (Ref. 1), S. Gay (Zurich, Switzerland) pointed out that gene transfer could be used to target these cells to inhibit their activation through interference with the mitogen-activated protein kinase (MAPK) pathway; determine the key enzymes that regulate the destruction of cartilage and bone; prevent the inhibition of apoptosis; and inhibit potent inflammatory cytokines.

Novel genetic targets

T. Pap (Magdeburg, Germany) discussed data concerning novel ribozymes that cleave the mRNAs encoding cathepsin L and collagenase [matrix metalloprotease 1 (MMP-1)], as well as an antisense construct directed against the membrane-type MMP (MT-MMP1), to determine the potential contribution of these proteinases in joint destruction. W. van der Laan (Leiden, Germany) showed that the transfer of cell-surface-targeted plasmin inhibitor ATF.BPTI [a hybrid protein of bovine pancreatic trypsin inhibitor (BPTI) and the amino-terminal fragment of urokinase plasminogen activator (ATF)] or tissue inhibitors of MMPs (TIMP-1 and TIMP-3) inhibited the invasion of cartilage by fibroblasts[2]. A novel approach was used by J. Mountz (Birmingham, AL, USA) to modulate the tumor necrosis factor α (TNF-α)-mediated apoptosis of synovial fibroblasts from RA patients by transfection with an adenovirus expressing a dominant-negative form of serine/threonine protein kinase B (PKB)[3].

In the search for new ways of interfering with the cytokine network that operates in collagen-induced arthritis (CIA), I.H. Tarner (Stanford, CA, USA) used retrovirally transduced

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IL-18 Receptors, Their Role in Ligand Binding and Function: Anti-IL-1RAcPL Antibody, a Potent Antagonist of IL-18[1]

Reno Debets, Jackie C. Timans, Tatyana Churakowa, Sandra Zurawski, Rene de Waal Malefyt, Kevin W. Moore, John S. Abrams, Anne O'Garra, J. Fernando Bazan, and Robert A. Kastelein[2]

IL-18 is critical in eliciting IFN-γ production from Th1 cells both in vitro and in vivo. Th1 cells have been implicated in the pathogenesis of autoimmune disorders, making antagonists of IL-18 promising therapeutics. However, specificity and binding characteristics of IL-18R components have only been superficially explored. In this study, we show that IL-1R related protein 1 (IL-1Rrp1) and IL-1R accessory protein-like (IL-1RAcPL) confer responsiveness to IL-18 in a highly specific (no response to other IL-1 ligands) and unique manner (no functional pairing with other IL-1Rs and IL-1R-like molecules). Cotransfection with both receptor components resulted in expression of both low and high affinity binding sites for IL-18 (K_d of 11 and 0.4 nM, respectively). We prepared anti-IL-1RAcPL mAb TC30-28E3, which, in contrast to soluble R proteins, effectively inhibited the IL-18-induced activation of NF-κB. Quantitative PCR showed that Th1 but not Th2 cells are unique in that they coexpress IL-1Rrp1 and IL-1RAcPL. mAb TC30-28E3 inhibited IL-18-induced production of IFN-γ by Th1 cells, being at least 10-fold more potent than anti-IL-18 ligand mAb. This study shows that IL-1RAcPL is highly specific to IL-18, is required for high affinity binding of IL-18, and that the anti-IL-1RAcPL mAb TC30-28E3 potently antagonizes IL-18 responses in vitro, providing a rationale for the use of anti-IL-1RAcPL Abs to inhibit Th1-mediated inflammatory pathologies. *The Journal of Immunology,* **2000, 165: 4950–4956.**

Interleukin-18 is a pleiotropic cytokine initially discovered as an IFN-γ inducing factor derived from liver cells (1, 2). Other biological activities of IL-18 include its ability to induce the production of inflammatory mediators, to enhance the cytotoxic activity of NK cells and T cells, and to augment the differentiation and activation of Th1 cells (1, 3–7). IL-12 and IL-18 single and double-knockout mice suggest that IL-18 is functionally closely related to IL-12 and plays a fundamental role in Th1 cell responses (8). However, IL-18 is predicted to fold as a β-rich trefoil, which is typical for IL-1 ligands (7). In fact, both IL-1β and IL-18 are produced as inactive precursors, which require cleavage by caspase-1 for secretion and activity (9, 10). Furthermore, IL-18 acts via the receptors IL-1R related protein 1 (IL-1Rrp1)[3] (IL-1R5) and IL-1R accessory protein-like (IL-1RAcPL) (IL-1R7) (11–13). Both receptors belong to the IL-1R family (see legend to Table I for a list of IL-1R family members and the numbering system used in this paper). IL-18 also activates signaling components which are involved in classical IL-1 signaling (7, 24).

The potent responses of IL-1α/β as well as IL-18 are tightly controlled by separate natural regulatory systems. IL-1 receptor antagonist (IL-1ra) and IL-1R2, for instance, antagonize the response to IL-1α/β at the ligand and (co)receptor levels (25–28), whereas the soluble IL-18 binding protein antagonizes IL-18 by preventing its access to its receptor (29, 30). Numerous studies have shown that perturbation of such control mechanisms contributes to the pathogenesis of inflammatory and infectious diseases and various cancers (31, 32), and, in the case of IL-18, particularly to endotoxin-induced liver injury and Th1-mediated autoimmune disorders (1, 33–37). Interference with the IL-18 ligand-receptor interaction has proven to be an effective strategy for the development of IL-18 antagonists. For instance, anti-IL-18 Abs protect mice primed with *Propionibacterium acnes* and challenged with LPS from liver damage and inhibit progression of experimental acute encephalomyelitis in rats (1, 34). The soluble IL-18 binding protein inhibits bacterial-induced IFN-γ production (29). Moreover, anti-IL-1R5 Ab reduces Th1 responses to LPS (38). Even though IL-1R5 is a functional component of the IL-18R, its binding affinity for IL-18 is relatively low (11). However, both high and low affinity binding sites for IL-18 were observed on IL-12-pretreated T and B lymphocytes (39). IL-1R7, a candidate receptor to explain this discrepancy, has not been analyzed in great depth with respect to its role in ligand-receptor interaction or its expression on inflammatory effector cells and not at all with respect to its potential as a target to inhibit inflammatory pathologies.

In this report, we show that in the context of the IL-1 system IL-1R7 is highly specific for the IL-18 response. IL-1R7 is furthermore required for high affinity binding of IL-18, and is coexpressed with IL-1R5 on Th1 but not Th2 cells. We prepared an anti-IL-1R7 mAb 28E3, wich effectively inhibited IL-18 responses in vitro. This result independently confirms the critical role of IL-1R7 in IL-18 action.

Materials and Methods

Biological reagents and cell culture

Recombinant human and mouse IL-1α and IL-1β were obtained from R&D Systems (Minneapolis, MN), and recombinant mouse IL-12 was obtained from BD PharMingen (Franklin Lakes, NJ). Recombinant human

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[3] Abbreviations used in this paper: IL-1Rrp1, IL-1R related protein 1; IL-1RAcPL, IL-1R accessory protein-like; sR, soluble R; EST, expressed sequence tag; RLU, relative light unit.

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Table I. *IL-1-induced activation of NF-κB is highly specific at the receptor level*[a]

Receptors		Ligands			
1°	2°	IL-1α	IL-1β	IL-18	IL-1ra
R1	R3	+	+	−	−
	R4	−	−	−	
	R5	−	−	−	−
	R6	−	−	−	
	R7	−	−	−	
	R9	−	−	−	
	R10	−	−	−	−
R4	R3	−	−	−	−
	R5	−	−	−	−
	R6	−	−	−	
	R7	−	−	−	
	R9	−	−	−	
	R10	−	−	−	−
R5	R3	−	−	−	
	R6	−	−	−	−
R5	R7	−	−	+	−
	R9	−	−	−	
	R10	−	−	−	
R6	R3	−	−	−	−
	R7	−	−	−	
	R9	−	−	−	
	R10	−	−	−	−
R7	R3	−	−	−	−
	R9	−	−	−	
	R10	−	−	−	−
R9	R3	−	−	−	
	R10	−	−	−	
R10	R3	−	−	−	

[a] The following IL-1R and IL-1R-like molecules (except IL-1R8) were used for transfections: IL-1RI (termed IL-1R1) (14); IL-1RII (IL-1R2) (15); IL-1R accessory protein (IL-1RAcP) (IL-1R3) (16); T1/ST2 (IL-1R4) (17, 18); IL-1R-related protein 1 (IL-1Rrp1) (IL-1R5) (19) and 2 (IL-1R6) (20); IL-1RAcPL (IL-1R7) (12); single Ig domain IL-1R-related protein (SIGIRR) (IL-1R8) (21); IL-1R accessory protein-like (IL-1RAPL) (IL-1R9) (22); and IL-1R10 (T. R. Sana, R. Debets, J. C. Timans, J. F. Bazan, and R. A. Kastelein. *Submitted for publication*). The receptor numbering is in keeping with our previously proposed numbering system (23). IL-1R-induced NF-κB activities in Jurkat cells were assayed as described in the legend to Fig. 1*A*. A plus sign was assigned when IL-1 stimulation of Jurkat IL-1R cotransfectants resulted in luciferase values, normalized for β-galactosidase activities, which were at least 3 times higher than background values (i.e., medium only). Data are from one of two to three independent experiments with similar results.

IL-18 and IL-1ra were produced at DNAX. Anti-mouse IL-18 mAb C18.6 and the corresponding isotype control Ab were obtained from BD PharMingen. The Cop5 cell line was maintained in DMEM supplemented with 5% FBS and 10 μg/ml ciprofloxacin (Miles, Kankakee, IL). The Jurkat E6.1 cell line was maintained in RPMI 1640 medium supplemented with 10% FBS, and 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Paisley, U.K.). The HDK-1 Th1 clone was cultured as described previously (40).

Brief description on cloning of human and mouse IL-1R7

Searching the public murine expressed sequence tag (EST) database with the intracellular portion of murine IL-1R3 revealed EST μ27d04.r1 (GenBank accession no. AA203986), which was ordered from the IMAGE consortium. A full-length mouse IL-1R7 containing clone was pulled out a spleen library using primers (5′-GCAGCAGTGAATCTTGCCTTGGTT and 3′-GCAGAGGTCTGGAGTATTGC) covering the latter half of the Toll Homology Domain (i.e., blocks 5–10) (23) (Genome Systems, St. Louis, MO). The search for the human homologue of murine R7 started off by the finding of two human ESTs (HGBFD13R, HCFCJ10R) in the Human Genome Sciences database (HGS, Rockville, MD) using the mouse EST as a query. The insert of clone HGBFD13R was ^{32}P-labeled and used to probe a cDNA library of activated HY06 cells (a Th1 clone). Analysis of positive clones extended the sequence up to the second Ig domain, and again the full-length sequence was obtained by screening a spleen library with primers (5′-TGATGTAGCCTGTTGTGTCAAGATG and 3′-GTACTGAGACTTCCCACTCTAGGTC) covering the latter half of the second and the complete third Ig domain (Genome Systems).

Expression vectors

For mammalian expression, vectors encoding full-length human and mouse IL-1R1, mouse IL-1R3, mouse IL-1R4, human and mouse IL-1R5, mouse IL-1R7, human IL-1R9, and human IL-1R10 were constructed by inserting PCR-generated cDNA fragments into pME18S (41). Human IL-1R6 was a generous gift of Dr. R. A. Maki (Neurocrine Biosciences, San Diego, CA). Both human IL-1R6 and human IL-1R7 cDNA were subcloned directly into pME18S. The cDNAs encoding extracellular parts of mouse IL-1R5 and IL-1R7 were fused to a C-terminal Ig cDNA module and Etag, respectively, via PCR and inserted into pCDM8 (Invitrogen, Carlsbad, CA). The reporter gene plasmid pNF-κB-Luc (Stratagene, La Jolla, CA) contains five NF-κB sites and a basic promoter element to drive luciferase expression, and pRSV-βGal results in constitutive expression of β-galactosidase.

Protein expression and purification of mouse soluble IL-1R5 and IL-1R7

Cop5 cells (10^7) were transfected by electroporation (200 V, 960 mF) with 15 μg of either mouse soluble IL-1R5 pCDM8.Ig or IL-1R7 pCDM8.Etag expression constructs (purified via Endo Free kit; Qiagen, Hilden, Germany). Cells (from five electroporations) were cultured in 1-liter culture medium using one 10-tray Cell Factory (Nunc, Kamstrup, Denmark). Seven to ten days posttransfection, supernatants were harvested by filtration and run over HiTrap columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden) coupled with either protein A or anti-Etag mAb, respectively. Soluble R5 (sR5) and sR7 proteins were eluted with 0.1 M glycine, pH 3.0, neutralized by addition of Tris-HCl, pH 8.0, and dialyzed vs PBS. The protein content was quantified by ELISA according to standard procedures. Cells transfected with empty constructs or with constructs containing an irrelevant insert provided mock controls. Endotoxin levels were determined using the *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD) and were <2.5 endotoxin units per 100 μg of protein. Protein samples were stored at 4°C.

Preparation of rat anti-mouse IL-1R7 mAb

Rat anti-mouse IL-1R7 mAbs were produced from splenocytes of an 8-wk-old female Lewis rat (Harlan Sprague-Dawley, Indianapolis, IN) immunized with mouse sIL-1R7:Etag fusion protein. The rat was primed i.p. with 25 μg of fusion protein in CFA and boosted three times i.p. with 10 μg (day 25), 5 μg (day 40), and 10 μg (day 54) in IFA, respectively. The final boosts were done both i.v. and i.p. at day 83 with 10 μg of fusion protein in saline solution and IFA, respectively. Splenocytes were fused at day 87 with the mouse myeloma P3X63-AG8.653 using PEG 1500 (Roche Diagnostics, Mannheim, Germany). Hybridoma supernatants were screened by indirect ELISA, Western blot, and FACS analysis. Selected positive hybridoma lines were subcloned and grown in serum-free medium supplemented with SITE (Sigma, St. Louis, MO). Abs were purified via HiTrap SP and Q columns (Amersham Pharmacia Biotech), and screened for their ability to inhibit IL-18-induced biological responses such as activation of NF-κB and production of IFN-γ.

Radiolabeling of IL-18

Human IL-18 was radioiodinated using the Bolton-Hunter Reagent, and subsequently purified to homogeneity via ion-exchange chromatography (Mr. G. Brown, Amersham Pharmacia Biotech). Specific radioactivity of the preparations was about 0.1 μCi/ng protein. The labeled IL-18 preparations were tested for their capacity to induce production of IFN-γ by the human NK cell line NKL.23.

IL-18 receptor binding assay

293-T cells were transiently transfected with human IL-1R5 and IL-1R7 pME18S expression constructs (20 μg each/10^7 cells) using $Ca_3(PO_4)_2$ (5 Prime→3 Prime, Boulder, CO) according to the supplier's protocol. Transfectants were used for receptor binding studies 3 days posttransfection. Cells were dislodged with 0.1% EDTA, rinsed extensively and suspended in PBS supplemented with 1% BSA and 0.1% NaN_3 (binding medium). The binding reactions were performed with 2.5×10^6 cells for 1 h at 4°C in 100 μl of binding medium containing 20 pM-10 nM ^{125}I-IL-18 with or without 1 μM unlabeled human IL-18. Subsequently, the mixtures were

layered over 150 μl of phthalate oil (dibutylphthalate:bis(2-ethylhexyl)phthalate, 3:2; Fluka, Milwaukee, WI) and centrifuged at 5000 × *g* for 10 min at 4°C. Cell-bound and cell-free radioactivity were measured in a gamma counter. Receptor binding data were analyzed by the Scatchard Coordinate System.

Quantitation of mRNA expression

A panel of DNAX mouse cDNA libraries, derived from various tissues and cellular sources (42, 43), was used for Taqman-PCR analyses. The Th1 and Th2 cells were prepared as described previously (44). RNA was isolated by the guanidium/phenol method (45). Reverse transcriptase reactions were performed with SuperScriptII (Life Technologies) according to the supplier's instructions, except that random hexamers (Promega, Madison, WI), at a final concentration of 1.25 μM, were added to the reaction. cDNAs (50 ng per reaction) were analyzed for the expression of IL-1R5 and IL-1R7 genes by the fluorogenic 5′-nuclease PCR assay (46), using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). Reactions were incubated for 2 min at 50°C, denatured for 10 min at 95°C and subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min followed by denaturation at 95°C for 15 s. The amplicons for IL-1R5 (nt 272–344, with numbers starting at the initiator codon) and IL-1R7 (nt 11–82) were analyzed with FAM-labeled probes. Total cDNA quantities were prenormalized to a household gene (GAPDH), and values were expressed as femtograms per 50 ng total cDNA.

Reporter assay

For reporter gene assays, Jurkat E6.1 cells (4×10^6) were transiently transfected by electroporation (300 V, 960 mF) with 2 μg of pNF-κB-Luc reporter gene plasmid, 0.5 μg of pRSV-βGal and 4 μg of each IL-1R-encoding cDNA (except when indicated otherwise). Twenty hours post-transfection, cells were stimulated with 20 ng/ml of human IL-1α, IL-1β, IL-18, or IL-1ra for 6 h. Cells were lysed using Reporter Lysis Buffer (Promega), and luciferase and β-galactosidase activities were assessed using Luciferase Assay Reagent (Promega) and Galacto-Light Kit (Tropix, Bedford, MA), respectively. Luciferase activities (in relative light units (RLU)) were normalized on the basis of β-galactosidase activities. Inhibition studies of mouse IL-18-mediated activation of NF-κB were performed under suboptimal conditions. Cells were transfected with 50 ng IL-1R5/7 cDNAs, and stimulated with 1 ng/ml IL-18 with or without anti-IL-1R7 hybridoma supernatants and sIL-1R5 and/or IL-1R7 protein preparations at varying concentrations. Hybridoma supernatants were concentrated using 50 kDa M_r cutoff filters (Millipore, Bedford, MA).

IL-18 bioassay

The Th1 clone HDK1 (40) was used to assay IL-18-induced production of IFN-γ at least 10 days after Ag stimulation, and after culture in medium with IL-2 alone (7). In short, cells were seeded in 96-well plates at 5×10^4 cells/well in the presence of suboptimal amounts of IL-18 (2 ng/ml) and IL-12 (0.2 ng/ml), with or without purified mAbs at the indicated concentrations. Supernatants were collected at 48 h and assayed for IFN-γ using a two-site sandwich ELISA (47, 48) with a sensitivity of 125 pg/ml.

Results

IL-1R5 and IL-1R7 are highly specific for IL-18-induced activation of NF-κB

To study the specificity of the IL-18R components for the IL-18 response within the IL-1 system of receptors and ligands, we matched all possible pairs of IL-1Rs and IL-1R-like molecules with known IL-1 ligands, using luciferase as a reporter for receptor-mediated activation of NF-κB (see Table I). These experiments revealed that the IL-1R pair IL-1R5/7 is solely responsive to IL-18 (see Fig. 1*A*). In addition, the response to IL-18 is only observed when IL-1R5 is paired with IL-1R7, and not with any other combination of IL-1Rs containing either IL-1R5 or IL-1R7 (see Fig. 1*B*). The total set of IL-1(R) pairing data (Table I) shows that the IL-1R5/R7 pair is unique to the IL-18 response, whereas the IL-1R1/R3 pair is unique to the IL-1αβ response. Mock or single receptor transfectants never showed a ligand-induced response (data not shown).

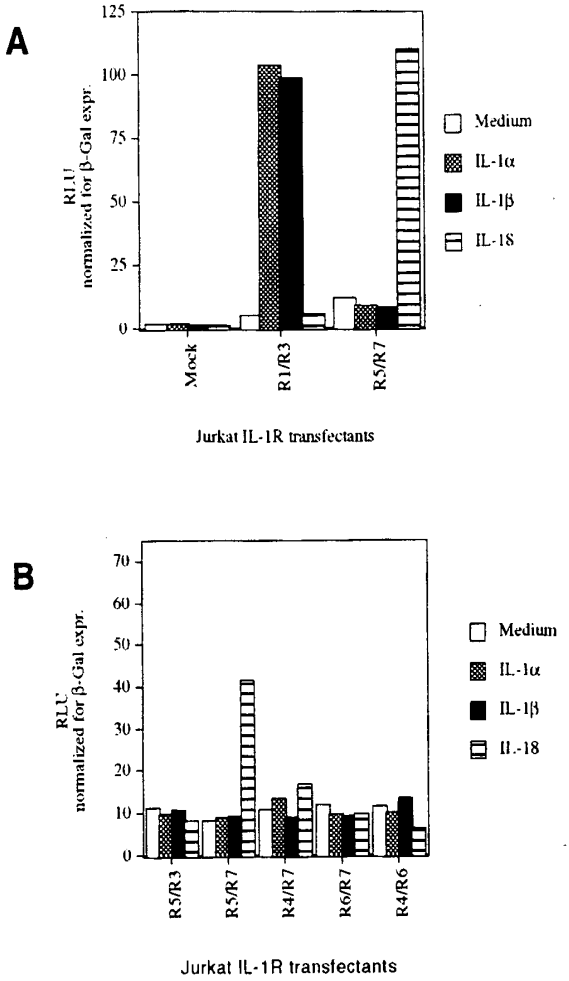

FIGURE 1. *A*, The IL-1R5/7 pair is specific for IL-18-induced activation of NF-κB. Jurkat cells (4×10^6) were transfected with 2 μg of pNF-κB-Luc reporter gene plasmid, 0.5 μg of pRSV-βGal, and 4 μg of each IL-1R plasmids (in pME18S, all human, except mouse IL-1R3) as indicated. Twenty hours after transfection, cells were left untreated or were stimulated for 6 h with all known human IL-1 ligands (20 ng/ml final). Luciferase activities were determined and normalized on the basis of β-galactosidase activities. Mock or single receptor transfectants did not give any luciferase response. Data shown are from one of five independent experiments with similar results. *B*, The IL-1R5/7 pair is unique for IL-18-induced activation of NF-κB. IL-1R-induced NF-κB activities in Jurkat cells were assayed as described in *A*. Data are from one of three independent experiments with similar results.

IL-1R5 and IL-1R7 cotransfectants provide low and high affinity binding to IL-18

To determine whether IL-1R7 contributes to the binding affinity of IL-18, binding studies on IL-1R5 and IL-1R7 double-transfectants were performed. We radiolabeled and purified human IL-18 via different methods and checked the integrity of the labeled molecule with an NK cell line-based bioassay. Studies showed that labeling on either tyrosine or lysine residues is succesful, but that purification via HPLC is detrimental for the biological activity of the cytokine, whereas other purification schemes did not affect the integrity of the cytokine (data not shown). We found labeling with the Bolton Hunter Reagent followed by purification using ion-exchange chromatography to work best. At least 40% of IL-1R5/7 transfected cells had to express protein, based on analysis of parallel transfectants with green fluorescent protein, for binding to be

successful. Expression of both human IL-1R5 and IL-1R7 by 293-T cells resulted in a dose-dependent specific binding of radioiodinated IL-18 (Fig. 2*A*). Scatchard analyses, as presented in Fig. 2*B*, revealed that expression of both receptors resulted in low-affinity binding (~4300 binding sites per cell with K_d: 11 nM) and high-affinity binding (~540 binding sites per cell with K_d: 400 pM).

Inhibition of IL-18-induced activation of NF-κB by anti-IL-1R7 mAb TC30-28E3, but not by soluble receptors

We prepared a panel of rat anti-mouse IL-1R7 hybridomas. Hybridoma supernatants were tested for their ability to inhibit IL-18-induced activation of NF-κB in IL-1R5/7-transfected Jurkat cells. Supernatants of 3 of 20 clones showed a dose-dependent inhibition of NF-κB activation, with the TC30-28E3 clone giving the strongest and most consistent inhibition (illustrated in Fig. 3*A*). Mouse sIL-1R5 and sIL-1R7 proteins did not affect the IL-18-induced activation of NF-κB (Fig. 3*B*).

Th1 but not Th2 cells uniquely express both IL-1R5 and IL-1R7 mRNA

To extend our analyses of mAb TC30-28E3 to responses mediated via physiologically expressed IL-18R components, we determined

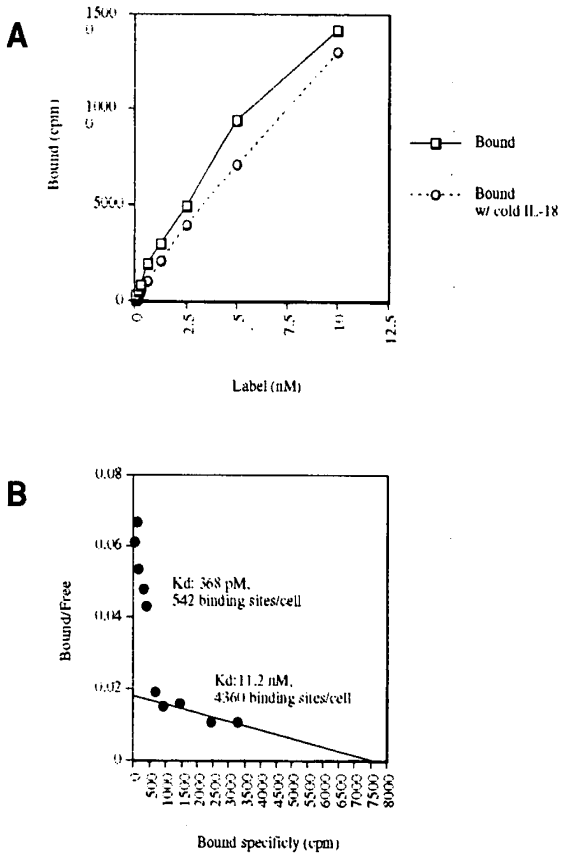

FIGURE 2. *A*, IL-1R5/7 cotransfectants specifically bind IL-18 in a dose-dependent fashion. 293-T cells transiently transfected with both human IL-1R5 and IL-1R7 were incubated (at 2.5×10^6 cells) for 1 h at 4°C with 20 pM-10 nM ^{125}I-IL-18. Nonspecific binding was measured by the addition of 1 μM unlabeled (cold) human IL-18. Specific binding is the difference between the total binding and binding with cold IL-18. *B*, IL-1R5 and IL-1R7 cotransfectants show low and high affinity binding to IL-18. Scatchard analyses were performed on specific binding data (*A*). The number of binding sites and the dissociation constants were calculated from the linear regression lines. Data are from one of two independent experiments with similar results.

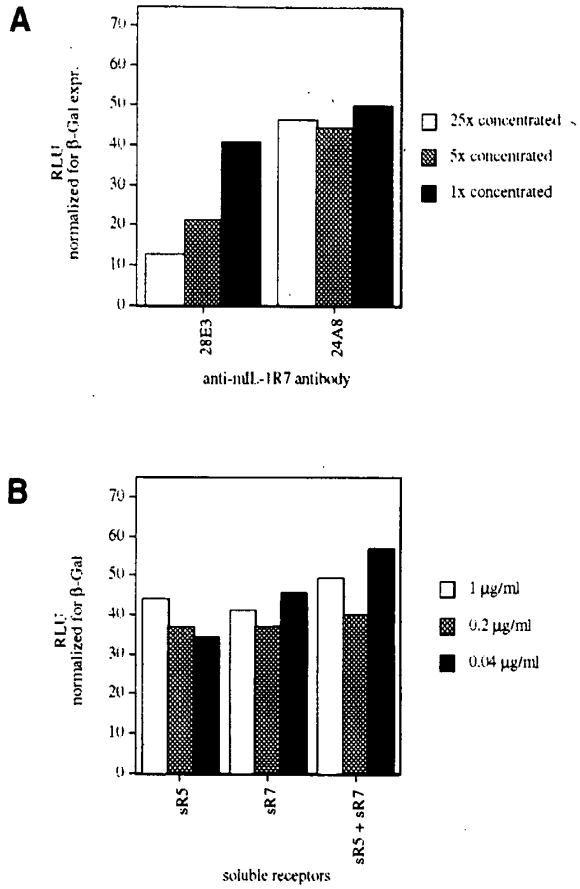

FIGURE 3. *A*, Anti-IL-1R7 mAb TC30-28E3 effectively inhibits IL-18-induced activation of NF-κB. IL-1R-induced NF-κB activities in Jurkat cells were assayed as described in *A*, with the following exceptions. Transfections were done with 50 ng mouse IL-1R5/7 cDNAs, and transfectants were stimulated with 1 ng/ml IL-18 with or without various concentrates of anti-mouse IL-1R7 hybridoma supernatants. A nonantagonizing anti-IL-1R7 mAb TC30-24A8 was used as a control. Luciferase values normalized for β-galactosidase activities were 48,000 and 3,200 RLU for mouse IL-18 and medium stimulations without anti-IL-1R7 mAbs, respectively. Preincubation of mAbs with IL-1R transfectants did not affect results. Data shown are from one of three independent experiments with similar results. *B*, Soluble IL-1R5 and IL-1R7 do not inhibit IL-18-induced activation of NF-κB. IL-1R-induced NF-κB activities in Jurkat cells were again assayed as described in *A*, except that mouse soluble IL-1R5 and/or IL-1R7 proteins were used as IL-18 antagonists at varying concentrations. Normalized luciferase values for mouse IL-18 and medium stimulations without soluble receptor proteins were as mentioned in *A*. Controls for fusion partners (Ig and E-tag) did not affect observed results. Also, preincubation of soluble receptors with IL-18 did not affect results. Data are from one of three independent experiments with similar results.

the RNA expression profile of IL-1R5 and IL-1R7 in a broad panel of cDNA libraries derived from various tissues and cell types. Quantitative PCR showed that these receptors were in fact coexpressed to significant levels almost exclusively on Th1 cells (Fig. 4). Th2 cells only expressed IL-1R7 mRNA to some extent, but not IL-1R5 mRNA.

Anti-IL-1R7 mAb TC30-28E3 is a potent antagonist of the IL-18-induced production of IFN-γ by Th1 cells

The Th1 clone HDK1, which coexpresses both IL-18 receptor components, was used to monitor the potential of anti-IL-1R7 mAbs to inhibit IL-18-induced production of IFN-γ. Monoclonal Ab TC30-28E3 again proved to be most effective in inhibiting this

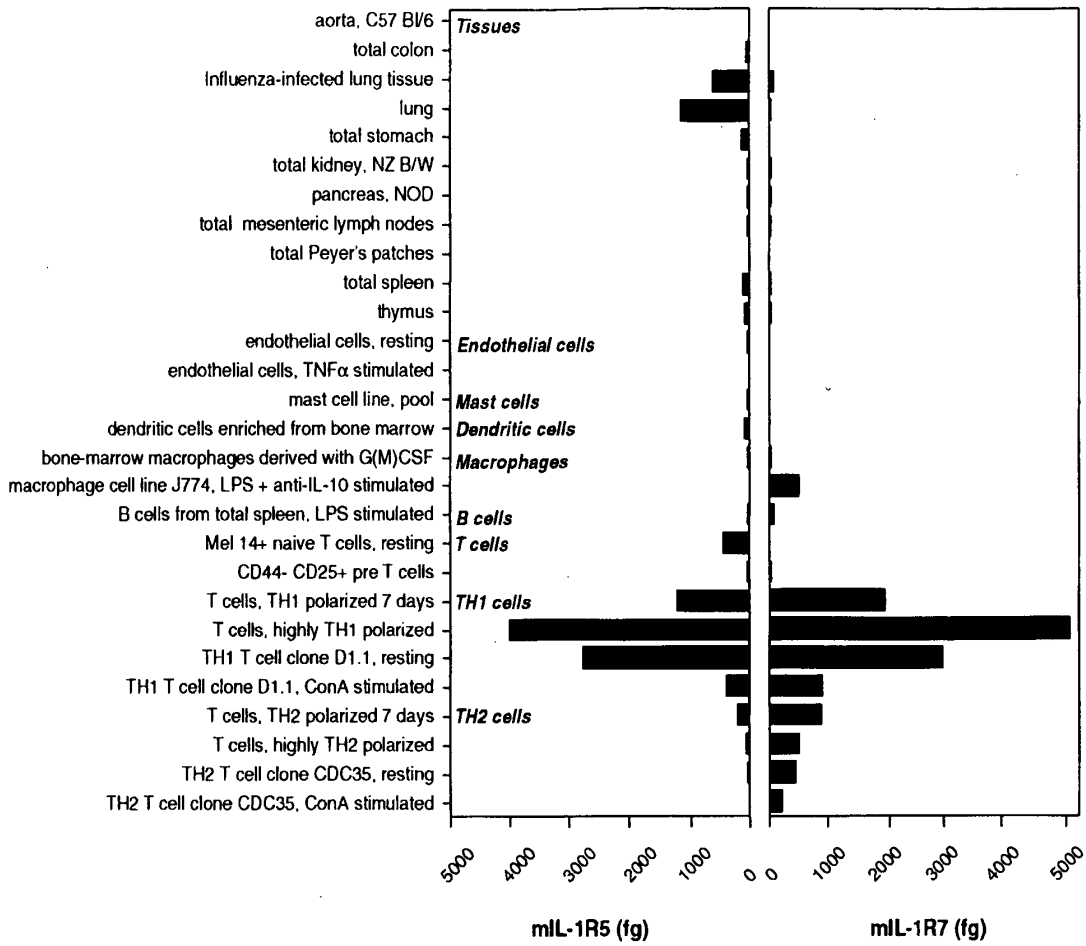

FIGURE 4. Th1 but not Th2 cells are unique in expressing high levels of both IL-1R5 and IL-1R7 mRNA. Taqman PCR was performed on a panel of human cDNAs made from various tissues and cell types. Cells were often left untreated or treated with different stimuli as indicated before RNA was isolated. Samples were analyzed for expression levels of mouse IL-1R5 and IL-1R7 by the fluorogenic 5′-nuclease PCR assay, using an ABI Prism 7700 Sequence detection System. The IL-1Rs were analyzed by FAM labeled probes. Expression levels were normalized and expressed as femtograms per 50 ng total cDNA.

response, in agreement with results obtained in the NF-κB activation assay. In fact, in the total set of Abs generated, there was a clear correlation between the effects on activation of NF-κB and the effects on IFN-γ production by either LPS-treated splenocytes or the Th1 clone HDK-1 (data not shown). Monoclonal Ab TC30-28E3 was purified and compared with anti-IL-18 ligand mAbs for their potency to inhibit IL-18-induced production of IFN-γ by HDK1 cells (Fig. 5). Anti-IL-18 mAb C18.6 showed similar levels of inhibition only at concentrations being at least 10-fold higher relative to mAb TC30-28E3 (Fig. 5). For a second anti-IL-18 mAb C18.7, the observed potency to antagonize the IFN-γ production by Th1 cells was even less than that of C18.6 (data not shown). Isotype control Abs did not affect the IL-18-induced production of IFN-γ by HDK-1 cells.

Discussion

Our studies focused on the specificity and binding characteristics of the IL-18R components, and the subsequent development and functional screening of anti-IL-1R7 mAb TC30-28E3. In this study, we report for the first time that: 1) both IL-18 receptor components (i.e., IL-1Rrp1 (IL-1R5) and IL-1RAcPL (IL-1R7)) are not only involved in the IL-18 response, but are in fact highly specific and unique for such a response; 2) IL-1R7 contributes to high affinity binding of IL-18; 3) Th1 but not Th2 cells coexpress both IL-18R components; and 4) the anti-IL-1R7 mAb TC30-28E3 potently inhibits IL-18-induced responses.

The IL-18R components both harbor extracellular Ig-folds and an intracellular domain homologous to the cytosolic part of the *Drosophila* Toll protein, characteristic of IL-1R and IL-1R-like molecules (see the legend to Table I for a list of IL-1R family members). The receptors of the IL-1 system typically comprise two separate receptor subunits, a ligand-binding receptor subunit and a signaling receptor subunit to establish a ligand-induced biological response. To study the specifity of IL-18R usage within the IL-1 receptor-ligand family, we functionally matched all possible pairs of IL-1Rs and IL-1R-like proteins with all known IL-1 ligands and IL-18. We found the IL-18-induced activation of NF-κB highly specific at the receptor level (Fig. 1). In fact, the total set of IL-1(R) pairing data (Table I) shows that the absence of redundancy of receptor usage applies to both known IL-1-type responses (i.e., IL-18 response via IL-1R5/7 and IL-1αβ response via IL-1R1/3). These observations suggest that the IL-1 family of ligands differ from the hemopoietic cytokines which signal through a common signaling receptor (i.e., common β, common γ, and the gp130 chain).

The IL-1α/β receptor expresses both low and high affinity binding sites for IL-1α/β (49). The majority of cellular binding sites expresses low-affinity binding for IL-1αβ, which are thought to depend on the expression of IL-1R1 alone (K_d about 1 nM). High-

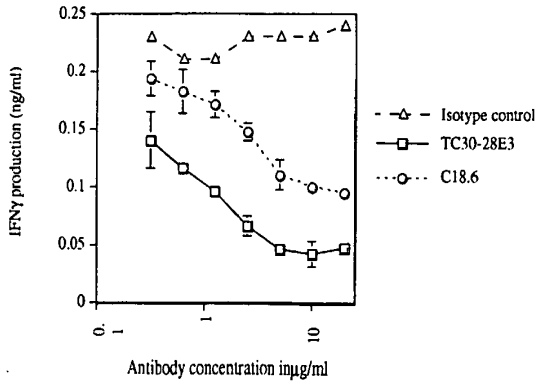

FIGURE 5. Anti-IL-1R7 mAb TC30-28E3 is a potent antagonist of the IL-18-induced production of IFN-γ by Th1 cells. IL-18-induced production of IFN-γ was assayed using clone HDK1. Cells were cultured in the presence of IL-18 (2 ng/ml) and IL-12 (0.2 ng/ml), with or without purified anti-IL-1R7 mAb TC30-28E3, anti-IL-18 mAb C18.6 or their corresponding isotype control Abs at the indicated concentrations. Supernatants were collected at 48 h and assayed using an IFN-γ ELISA. The concentration of IFN-γ without mAb was 0.317 ± 0.115 ng/ml. The isotype control Abs had a negligible effect on the production of IFN-γ, which is shown for one representative Ab. Data are presented as mean ± SD of triplicates of one experiment of two with similar results.

affinity binding to IL-1$\alpha\beta$ (K_d about 0.02–0.25 nM), making up at most 10% of the total number of binding sites per cell, however, is thought to depend on the coexpression of both IL-1R1 and IL-1R3 (16). To date, binding studies with IL-18 are limited. Analysis of a whole array of human leukemic cell lines (including various T and B cell leukemias) revealed that only a few cell lines showed specific binding to IL-18 (11). The IL-18 binding characteristics of one such cell line (i.e., the Hodgkin's disease cell line L428) were similar to those of IL-1R5 transfectants and pointed to low-affinity binding by IL-1R5 alone (2–5 × 10^4 sites per cell with K_d 18.5–46.3 nM) (11). IL-12 pretreated mouse T and B cells show an increased expression of IL-1R5, and expressed both low and high affinity binding for IL-18 (for T cells: 5500 sites per cell with K_d 31.4 nM, and 405 sites per cell with K_d 430 pM, respectively) (39). IL-1R7 seems not be able to bind IL-18 (12), but its possible role in increasing the affinity of IL-1R5 for IL-18 is unclear. To address this issue, we performed binding studies with cells expressing both IL-1R5 and IL-1R7. Coexpression of both IL-1R5 and IL-1R7 indeed resulted in low and high affinity binding of IL-18 (Fig. 2), with binding properties similar to those found for IL-12 pretreated T cells. These data suggest that IL-1R5 and IL-1R7 form a complex that functions as a high affinity binding site for IL-18.

The observations that IL-1R7 is highly specific for the IL-18 response and is required for high affinity binding to IL-18, suggested the utility of anti-IL-1R7 mAbs to block IL-18-mediated responses. We prepared a panel of rat anti-mouse IL-1R7 mAbs, of which mAb TC30-28E3 effectively inhibited the IL-18-induced activation of NF-κB (Fig. 3). Induction of direct cell death by the IgG2a isotype mAb TC30-28E3 via complement-mediated cytotoxicity was ruled out (tested by trypan blue dye exclusion, not shown). Soluble R5 and R7 proteins did not affect the IL-18-induced activation of NF-κB. This is somewhat unexpected because sR1 and sR2 proteins were reported to inhibit IL-1-mediated responses in vitro and in vivo (50, 51). Moreover, sR5 but not sR7 protein has been found to bind IL-18 in solution (12). However, the binding affinity of IL-1R5 for IL-18 is at least 20-fold lower than the binding affinity of IL-1R1 for IL-1 (11, 49), and the binding affinity of sR5 for IL-18 may even be lower than that of membrane-bound R5. BIAcore biosensor studies confirmed that sR5 but not R7 bound IL-18 only to a weak extent (data not shown). Poor binding properties of sR5 and R7 probably account for their inability to block IL-18-mediated activation of NF-κB.

Others have reported that IL-1R5 is expressed exclusively on Th1 cells relative to Th2 cells (38, 39). We have extended this finding by showing that Th1 cells, among a broad panel of various tissues and cell types, are unique in coexpressing significant levels of both IL-1R5 and IL-1R7 mRNA (Fig. 4). Th1 but not Th2 cells also express IL-12Rβ2 (52), which is selectively lost in early Th2 cells (53). Whether IL-18R subunits are also selectively lost in early Th2 cells needs yet to be determined. We extended our analyses of mAb TC30-28E3 to responses mediated via endogenously expressed IL-18 receptors by Th1 cells. Monoclonal Ab TC30-28E3 potently inhibited LPS-induced production of IFN-γ by spleen cells (not shown) as well as IL-18-induced production of IFN-γ by the Th1 clone HDK1, and proved to be a more potent antagonist than anti-IL-18 mAb (Fig. 5). The TC30-28E3 mAb and the anti-IL-18 mAb used in this study may bind to their respective target Ags with different affinities or IL-1R7 may in fact represent a more efficient target than IL-18 to block IL-18-mediated responses. The synergy between IL-18 and IL-12 to induce production of IFN-γ, which is probably the result from reciprocal upregulation of their receptors (38, 54) and the use of distinct signaling pathways to enhance IFN-γ gene transcription (55), depends only partly on the activation of NF-κB (56). mAb TC30-28E3 therefore most likely blocks the ability of IL-18 to activate additional transcription factors such as AP-1 and possibly others. Our in vitro data are in line with preliminary in vivo data, which show that mAb TC30-28E3, especially in combination with anti-IL-12 mAb, abrogates the clinical progression of Th1-mediated diseases, such as *Listeria* infection (A.O., manuscript in preparation) or *Legionella* infection.[4]

Taken together, our study provides in vitro evidence that IL-1R7 confers responsiveness to IL-18 in a highly specific and unique manner, is required for high affinity binding of IL-18, and is coexpressed with IL-1R5 on Th1 cells only. Moreover, the anti-IL-1R7 mAb TC30-28E3 has properties highly suited to testing the therapeutic utility of anti-IL-1R7 Abs in inhibiting Th1-mediated pathologies.

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Anti-interleukin-1 therapy in rh umatic dis ases

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Recent research has shown that in the processes of rheumatoid arthritis (RA), interleukin (IL)-1 is one of the pivotal cytokines in initiating disease, and the body's natural response, IL-1 receptor antagonist (IL-1Ra), has been shown conclusively to block its effects. In laboratory and animal studies inhibition of IL-1 by either antibodies to IL-1 or IL-1Ra proved beneficial to the outcome. To date, two large well-controlled studies in patients with RA led to the conclusion that IL-1Ra is clinically effective and that it slows progression of bone damage as measured radiographically. Being a specific, selective inhibitor of the IL-1 pathway, IL-1Ra could constitute an important new approach to treating patients with RA that significantly reduces the signs and symptoms of the disease, reduces joint destruction and up to now has proved safe and well tolerated. Curr Opin Rheumatol 2001, 13:170–176

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Abbreviations

ACR	American College of Rheumatology
IL-1	interleukin-1
IL-1Ra	interleukin-1 receptor antagonist
IRAP	interleukin-1 receptor antagonist protein
MMP	matrix metalloproteinases
PGE_2	prostaglandin E_2
TNF-α	tumor necrosis factor-α
ICE	interleukin-converting enzyme
MTX	methotrexate
RA	rheumatoid arthritis

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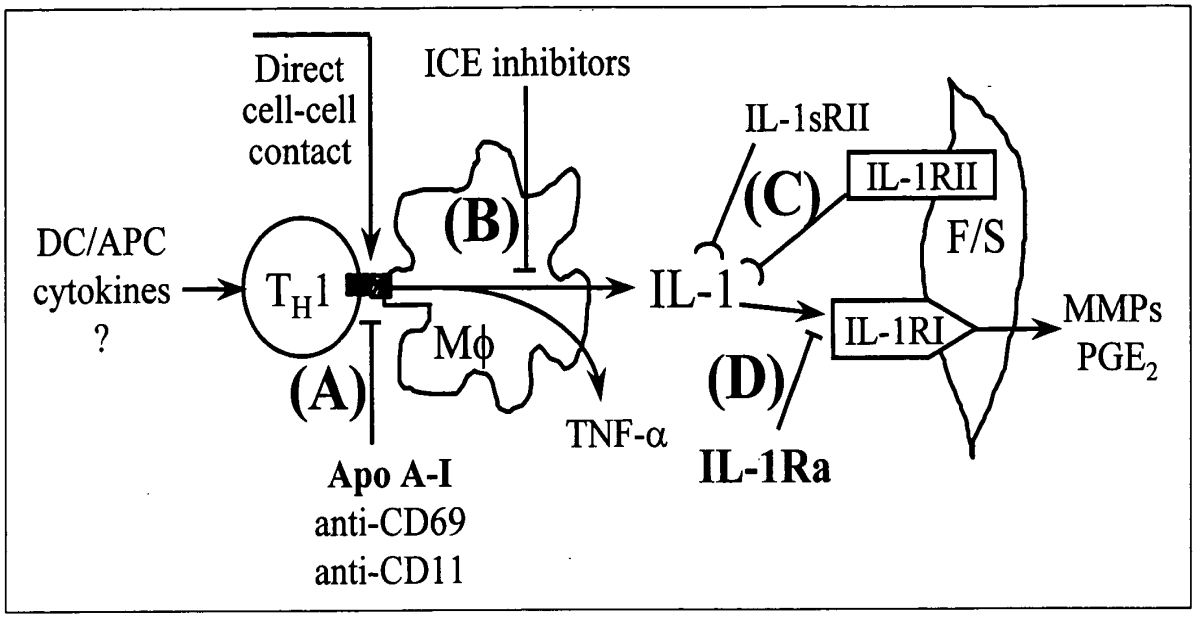
Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown cause, which is characterized by synovial inflammation and joint destruction. Numerous observations support the fact that tissue destruction and resulting disability in RA are partly the result of extracellular matrix degradation by proteolytic enzymes, including matrix metalloproteinases (MMPs) and the release of the mineral phase (Ca^{2+} release) by prostaglandin E_2 (PGE_2). The production of MMPs and PGE_2 is essentially induced by the proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), which play a major part in RA pathogenesis, according to *in vitro* experiments with cultured cells and in animal models. IL-1 and TNF-α are mainly produced by monocyte-macrophages, which are activated by direct contact with stimulated T cells at the inflammatory site [1–4]. Indeed, based on the premise that T lymphocytes play a pivotal role in the pathogenesis of chronic inflammatory diseases, we demonstrated that direct cell-cell contact with stimulated T lymphocytes is a major stimulus triggering the production of large amounts of TNF-α and IL-1β in monocytes [1,2,5•]. However, the identity of the ligands on plasma membrane of stimulated T cells that trigger the signaling of monocyte-macrophages as well as that of the counter-ligands on monocytes is still elusive. In the human system, some of the signaling may be attributed to β2-integrins, CD69, CD23, LAG-3, and CD40-CD40L [5•,6–12]. Membrane-associated TNF-α and IL-1 do not play a crucial part in this particular cellular interaction, in contrast with their significant role in activation processes induced by stimulated T cells in human fibroblasts/synoviocytes or microvascular endothelial cells [1,2,13,14].

Interleukin-1 in rheumatoid arthritis

In RA, monocyte-macrophages accumulate at the cartilage/pannus junction. In contrast with TNF-α, which is predominantly detected in the early stages of disease, both IL-1α and IL-1β are detected long after the onset of RA [15–17], thus justifying the use of IL-1 blocking therapy in all stages of the disease. The importance of IL-1 as a key mediator of bone resorption and cartilage destruction in RA has been well established, as reviewed in [18], and it may be controlled at several levels (Fig. 1). IL-1 production is strongly induced in monocyte-macrophages upon direct cellular contact with stimulated T lymphocytes. The latter mechanism is blocked by apolipoprotein A-I, which is likely to interact

Figure 1. Possible levels of IL-1 blockade in rheumatoid arthritis inflammatory cascad



(A) IL-1 can be inhibited by apolipoprotein A-I, anti-CD11, and anti-CD69 antibodies, which block its induction by direct contact with stimulated T cells (T). (B) The production of soluble IL-1β can be inhibited by blocking its processing by ICE. (C) IL-1 activity can be inhibited by binding to either soluble shed receptor or decoy receptor. (D) IL-1 activity can be blocked by binding of IL-1Ra to IL-1RI, thus hampering signal transduction.

with stimulating molecules expressed at the surface of stimulated T lymphocytes, thus potently inhibiting IL-1 production [19••]. The inhibition of this particular pathway is promising in therapeutic terms because it blocks not only the production of IL-1 but also that of TNF-α [19••]. Downstream, the generation of the active form of IL-1β is controlled by caspase-1 (interleukin-converting enzyme, ICE), whose activation by RIP-2 is prevented by ICEBERG [20]. However, the therapeutic use of intracellular proteic inhibitors such as ICEBERG requires intracellular expression and is therefore premature. Furthermore, ICEBERG inhibits the production of the soluble, active form of IL-1β without affecting the production of IL-1α, whose destructive functions might persist. Once produced, extracellular IL-1, membrane-associated, *ie,* IL-1α, or soluble, *ie,* IL-1β, binds to IL-1 receptors. The binding to the functional IL-1 receptor I (IL-1RI) is controlled by two different molecules, IL-1RII and IL-1 receptor antagonist (IL-1Ra).

The IL-1RII is expressed at the surface of cells that also express IL-1RI. However, the binding of IL-1 to IL-1RII does not transduce any signal, IL-1RII being a decoy receptor [21]. Furthermore, the shed form of IL-1RII, *ie,* soluble IL-1RII (sIL-1RII), also binds IL-1, thus diminishing the concentration of active IL-1 able to bind IL-1RI. Finally, IL-1 binding to IL-1RI is hampered by IL-1Ra. The latter inhibitor is unique in the cytokine network in that it binds competitively to IL-1RI but does not engage the formation of a high-affinity trimolecular complex with IL-1RI and IL-1R accessory protein [22]. The latter mechanism hampers the transduction of IL-1 signaling.

Interestingly, in the human system, the inhibitory activity of IL-1Ra is enhanced by sIL-1RII but hindered by the soluble, recombinant form of IL-1RI [23•]. IL-1Ra presents the advantage of inhibiting both IL-1α and IL-1β, which implies that it is potentially efficient at both early and late stages of the disease.

History of interleukin-1 receptor antagonist

Before the discovery of cytokine antagonists, the arguments for their existence were based on two facts: First, spontaneous regression of symptoms (such as fever) after the loss of biologic activity in biologic fluids can occur despite the persistence of proinflammatory cytokines. Second, mature "active" proinflammatory cytokines are present in biologic fluids, without any biologic activity being detected. The first evidence and proof of the concept of true receptor antagonists came from studying the biology of IL-1, when binding studies revealed a competitive mechanism at the receptor level [24••]. Until then, any biologic inhibitory activity found in culture supernatants or biologic fluids had simply been ascribed to inhibitory molecules. In contrast to IL-1Ra, molecules identified as specifically inhibitory to TNF-α resulted from the cleavage of soluble receptors [25–28]. Some of the milestones in the story of IL-1Ra

were inhibitory activity in urine of febrile patients and with monocytic leukemia [29•], IL-1 inhibitory activity in human monocytes [30•], IL-1 inhibitory activity in juvenile RA flares [31•], blocking of ligand binding to the receptor by interaction with the receptor [24•], purification of IL-1Ra [32], IL-1Ra blocking of IL-1-induced bone resorption and prostaglandin production [33], and complete characterization and cloning of IL-1Ra [34,35].

Interleukin-1 induces and exacerbates arthritis in animal models

Early work revealed that IL-1α or IL-1β injected into the knee joints of rabbits resulted within hours in the accumulation of leukocytes in synovial fluid and a substantial proteoglycan loss from articular cartilage [36,37]. In these studies, IL-1Ra inhibited the effects of IL-1 [38]. Furthermore, it was demonstrated that IL-1 and TNF-α were synergistic in inducing these responses [39••]. Feige *et al.* [40•] demonstrated that continuous infusion of IL-1α for 2 weeks into the knee joints of rabbits induced arthritic changes whose characteristics were reminiscent of RA. The results in rabbits were supported by the results of injection studies of IL-1 into rat knee joints, which showed that it is not only the dose of IL-1 but also the site and frequency of IL-1 administration that leads to the development of more chronic features of arthritis [41].

Infusion of IL-1β by implanted osmotic minipumps was found to accelerate the onset of collagen-induced arthritis in DBA/1 mice [42]. Also, daily IL-1β injections into 3.5-month-old MRL/lpr mice resulted in clinical arthritis in all of them within a few days, but not in noninjected mice [43]. In streptococcal cell wall–induced arthritis, where peptidoglycan–polysaccharide polymers isolated from cell walls of group A streptococci (PG-APS) are used to reactivate arthritis, intravenous IL-1 also resulted in an arthritic flare [44].

Mice deficient in IL-1Ra show both a higher incidence and greater severity of collagen-induced arthritis [45]. Interestingly, in IL-1Ra-deficient mice of the BALB/cA strain but not the C57BL/6J strain, chronic inflammatory arthropathy with clinical signs begins to develop at the age of 4 weeks [46]. By contrast, mice overexpressing IL-1Ra had a significant reduction in incidence and severity of collagen-induced arthritis [45]. It is worth noting that these mice showed only an insignificant reduction in B and T cell responses to collagen.

Interleukin-1 inhibition prevents inflammation, cartilage effects, and bone effects in animal models of arthritis

Interleukin-1 is a very potent cytokine, which is active at concentrations of picograms per milliliter, and only a few cellular receptors have to be occupied to trigger a response. Therefore, IL-1R has to be blocked completely at all times. Considering that the effects of IL-1Ra can be difficult to substantiate because of the very short half-life in mice and rats, researchers have used various strategies to overcome this dilemma: (1) four daily injections at 100 mg/kg [44], (2) continuous infusion using a catheter inserted into the jugular vein [47], (3) implantation of Alzet osmotic minipumps (Durect Corp., Cupertino, CA) [48••], or (4) use of single daily doses of 1000 mg/kg and higher (Feige *et al.*, unpublished data). As summarized in Table 1, IL-1Ra affects not only clinical symptoms but also the underlying disease processes such as cartilage and bone destruction [47,48••,49–58••].

Clinical experience with recombinant human interleukin-1 receptor antagonist

The clinical development of human recombinant IL-1Ra (anakinra), which differs from the native nonglycosylated human IL-1Ra only by the addition of an N-terminal methionine, has made it possible to study this protein for its therapeutic potential in RA [59••]. IL-1Ra has been extensively studied as monotherapy and demonstrated to be clinically effective [60]. In an initial study, 7-week dose ranging and dose frequency tested in 175 RA patients demonstrated that daily injections of IL-1Ra were more effective than weekly doses in improving individual components of the ACR response [61•]. On the basis of these results, the efficacy of IL-1Ra in RA was studied in considerably greater detail in a 24-week, multicenter, randomized, placebo-controlled, double-blind study [59••]. The 472 patients were randomized to one of four treatment groups: IL-1Ra 30 mg/d, IL-1Ra 75 mg/d, IL-1Ra 150 mg/d, or placebo. After 24 weeks of treatment, significantly more subjects (43%) who received IL-1Ra 150 mg/d met the American College of Rheumatology (ACR) criteria for a 20% response than did subjects who received placebo. Statistically significant improvements compared with placebo were observed at 24 weeks in the IL-1Ra 150 mg/d group for all six clinical parameters measured, including number of swollen joints, investigator assessment of disease activity, patient assessment of disease activity, pain score, and duration of morning stiffness [59••]. Importantly, patients continued to maintain their response when treated for an additional 24 weeks.

Effects of interleukin-1 receptor antagonist on joint damag

Serial hand radiographs were taken immediately before and after 24 weeks of treatment in 347 of the 472 patients (74%) entering the randomized clinical trial [59••]. The radiographs were analyzed according to the Larsen method [59••,62]. The mean Larsen score in the placebo group increased by 6.4 from 15.4 at base-

Table 1. Inhibition of arthritis by inhibiting interleukin-1

Model	Animal species	Treatment	Maximum dosage of IL-1Ra used	Result	Reference
IL-1Ra					
SCW arthritis	Lewis rat	4x daily ip	8 mg/kg	55% inhibition of paw swelling	[44]
Collagen-induced arthritis	DBA/1 mouse	daily ip	300 µg/mouse	Delay of onset and some reduction of severity	[49]
Antigen-induced arthritis	New Zealand white rabbit	4x daily ip	4 mg/kg	Reversal of synovial fibrosis. However, only minor effects on joint swelling, leukocyte accumulation, or cartilage proteoglycan loss	[50]
Collagen-induced arthritis	DBA/1 mouse	Osmotic minipumps from day 34	1.2 mg/d	Treatment after onset results in reduction of positive joints, synovial infiltrate and cartilage destruction.	[48••]
Antigen-induced arthritis	New Zealand white rabbit	2 hours before challenge iv	20 mg/kg	40% inhibition of proteoglycan loss	[51]
Collagen-induced arthritis	Lewis rat	Daily sc	100 mg/kg in HA	78% inhibition of inflammation (as well as bone effects)	[52]
Collagen-induced arthritis	Lewis rat	Continuous infusion	5 mg/kg/h	90% inhibition of inflammation (as well as bone effects)	[47]
Anti-IL-1 antibody					
Collagen-induced arthritis	DBA/1 mouse	mAb 1400.24.17 3 x weekly ip	100 µg/mouse	Treatment from day 21: delay of onset of disease for about 2 weeks. Treatment from day 3: complete inhibition of disease until day 60 (end of study).	[53]
Collagen-induced arthritis	DBA/1 mouse	Polyclonal rabbit anti-IL-1α/IL-1β	0.6 mg/mouse	Start of treatment before onset results in complete inhibition of arthritis. Treatment after onset results in reduction of positive joints, synovial infiltrate and cartilage destruction.	[54]
Collagen-induced arthritis	DBA/1 mouse	Polyclonal rabbit anti-IL-1α/IL-1β	0.6 mg/mouse	IL-1β predominates, although Il-1α also plays a role in this type of arthritis.	[48••]
Soluble IL-1 type II receptors					
Antigen-induced arthritis	Dwarf Russian rabbit	(Bolus iv) followed by osmotic minipumps	40.2 µg/h	Marked and significant inhibition of knee swelling, significant reduction of joint erosions, no effect on inflammatory cell infiltration	[55]
Collagen-induced arthritis	DBA/1 mice	sIL-1RII producing cells sc day 10	Not applicable	Delay of onset, reduction of incidence and severity	[56]

IL-1, interleukin-1; ip, intraperitoneal; iv, intravenous; sc, subcutaneous.

line to 21.8 at 24 weeks. By contrast, the mean Larsen score in the patients receiving IL-1Ra increased by no more than 3.8. This represents a 41% reduction in the rate of radiographic joint damage compared with placebo in the individual IL-1Ra treatment groups [59••]. When radiographic joint damage was assessed according to the number of joints with erosions, an increase of 2.6 from 5.0 to 7.6 was observed in the placebo group. By contrast, the mean number of joints with erosions in the subjects treated by IL-1Ra increased by only 1.4 (P = 0.004 compared with placebo), representing a reduction of 46% [59••].

There was also a reduction in radiologic progression as measured by both Larsen and Sharp scores as early as 6 months, which persisted after an additional 6 months of follow-up [63]. At 24 weeks, by the Genant method, there was a significant reduction in the score for progression of joint space narrowing (JSN) and the total score (a combination of erosion and JSN) in all IL-1Ra treatment groups [63]. The scores by two methods correlated strongly for each individual time point, but much less strongly for disease progression, suggesting that IL-1Ra reduced radiologic progression of RA [63].

Clinical effects of interleukin-1 receptor antagonist in combination with methotrexate

Methotrexate (MTX) is the most commonly used conventional disease-modifying antirheumatic drug. Combination regimens associating MTX with other biologic agents such as etanercept or infliximab have been used successfully when the disease has remained active and/or progressed when MTX is used alone [64,65]. To further support the use of combination

therapy in RA, and to evaluate the efficacy and safety of MTX with IL-1Ra, a 24-week multicenter, randomized, placebo-controlled, double-blind study was performed in patients who continued to have active disease despite maximally tolerated doses of MTX [66].

Patients with moderate to severe active RA upon MTX treatment (disease duration 6 months or more to 12 years) were randomized into six groups: placebo or IL-1Ra (0.04, 0.1, 0.4, 1.0, or 2.0 mg/kg daily) administered in a single daily subcutaneous injection. The primary efficacy endpoint was the proportion of subjects who demonstrated an ACR 20 response at week 12. A total of 419 patients were enrolled in the study. Patient demographics and disease status were similar in the six treatment groups. The ACR 20 responses at week 12 in the five active treatment plus MTX groups demonstrated a statistically significant ($P = 0.001$) dose-response relation compared with the placebo plus MTX group [66]. The ACR 20 response rate in the IL-1Ra 1.0-mg/kg (46%, $P = 0.001$) and 2.0-mg/kg (37%, $P = 0.007$) dose groups was significantly better than that in the placebo group (19%). The ACR 20 responses at 24 weeks were consistent with those at 12 weeks. Similar improvements in IL-1Ra-treated subjects were noted in individual ACR components, erythrocyte sedimentation rate, duration of morning stiffness, onset of ACR 20 response, sustainability of ACR 20 response, and magnitude of ACR response. IL-1Ra was safe and well tolerated [66]. Injection site reaction was the most frequent adverse event noted; it led to premature study withdrawal in 7% (1.0 mg/kg) to 10% (2.0 mg/kg) of patients at higher doses. In a related report, subjects receiving IL-1Ra experienced rapid improvement in health-related quality-of-life parameters, which were related to the dose of the biologic agent. These studies suggest that combination therapy with IL-1Ra and MTX is effective and safe for patients with active RA [66].

Safety of IL-1Ra

In clinical trials to date, IL-1Ra has been well tolerated [67]. Injection site reactions have been the most frequently observed adverse event, particularly with 1 mg/kg and 2 mg/kg doses. The European Monotherapy Study found injection site reactions to be the most frequent, and there were no changes in creatinine clearance rates or liver enzyme levels [67]. Furthermore, the safety record of IL-1Ra in terms of risk of infection compares favorably with that of the current biologic treatments that target TNF-α [65]. IL-1Ra showed no evidence of immunosuppression, increased risk of infection, or malignancy in controlled clinical trials [59••,67].

Effects of interleukin-1 receptor antagonist on synovial tissue

Studies were made on synovial biopsy tissue obtained from RA patients treated with IL-1Ra [68]. Twelve paired biopsy samples were obtained before and after the randomized clinical trial of IL-1Ra and evaluated for cellular infiltration and adhesion molecule expression after immunohistochemical staining [59••]. This demonstrated that IL-1Ra treatment of RA resulted in reduced mononuclear cell infiltration of synovial membrane, which may represent *in vivo* inhibition of biologically relevant IL-1β–mediated pathogenic effects [68].

In a related study, the distribution of radiolabeled IL-1Ra in patients with RA was assessed to determine whether this cytokine is suitable for scintigraphic visualization of synovitis [69]. In patients with active RA, scintigraphy was performed after a single intravenous dose of [^{123}I]-IL-1Ra. The clearance and organ distribution of radiolabeled IL-1Ra were also studied. To assess whether radiolabeled IL-1Ra targets the synovial IL-1 receptors, the scintigraphic images obtained with IL-1Ra were compared with those obtained by the use of a nonspecific control agent. The authors concluded that radiolabeled IL-1Ra allows the visualization of synovitis in patients with RA. However, neither the imaging nor the autoradiographic studies indicated that radiolabeled IL-1Ra bound to specific IL-1 receptors [69].

Gene therapy with IL-1Ra

The University of Pittsburgh is developing MFG-IRAP, a retroviral vector carrying the human interleukin-1 receptor antagonist protein (IRAP). In preclinical studies, cDNA for potential treatment of arthritis MFG-IRAP gene therapy was effective in local gene delivery to synovial lining of joints and systemically to hematopoietic stem cells. Intraarticular expression of IRAP, although transient (4 to 6 weeks), was efficacious in several animal models of arthritis [70•]. The successful completion of these studies will be an encouragement to develop additional human gene therapies for arthritis and other orthopedic and rheumatic conditions.

Conclusions

Because it is a specific, selective inhibitor of the IL-1 pathway, IL-1Ra offers an important new treatment for RA that significantly reduces the signs and symptoms of the disease, reduces joint destruction, and is safe and well tolerated. However, the combination of IL-1Ra with other immunomodulatory molecules such as MTX or anti-TNF-α should result in better anti-inflammatory and antidestructive effects.

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